

# ATTACHMENT

## Original Contribution

# EFFECT OF TUMOR BLOOD FLOW MANIPULATIONS ON RADIATION RESPONSE

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The effect of anesthetics and several vasoactive agents on the blood flow, temperature, and radiation response of the solid murine KHT sarcoma was evaluated to better understand the relationship of tumor blood flow and the proportion of hypoxic cells. Tumor blood flow, assessed by  $^{133}\text{Xe}$  clearance, decreased by 59 and 24% following treatment with pentobarbital and urethane, respectively. In addition, both drugs reduced body and tumor temperature. Metaraminol, a sympathomimetic agent, and nitroglycerin also reduced tumor blood flow by 40 and 12%, respectively. Tumor radiation response following drug treatment was quantitated by an *in vitro* agar assay. Results indicate that drug-induced reductions in tumor blood flow generally result in an increase in the apparent tumor cell hypoxic fraction.

KHT sarcoma, Tumor blood flow, Radiosensitivity,  $^{133}\text{Xe}$  clearance, Anesthetics, Vasoactive agents, Tumor temperature.

## INTRODUCTION

A considerable amount of effort in radiobiological research has been directed towards studies of hypoxic cells in solid tumors. Blood flow to the tumor is responsible for cell oxygenation and therefore would be expected to influence the proportion of hypoxic tumor cells. Agents which alter tumor blood flow would thus be expected to alter the hypoxic cell fraction. Several authors have reported that anesthesia alters the response of tumor cells to irradiation; the majority of studies indicate a radioprotective effect.<sup>3,17,20-23,27</sup> Alterations in tumor blood flow which occur during anesthetic administration<sup>4,10,12,28</sup> may contribute to the increased hypoxic cell fraction. It might also be expected that treatment with vasoactive agents would affect the apparent hypoxic cell fraction and exhibit either radioprotective or radiosensitization effects.

In the current study we describe the effect of anesthetics and vasoactive agents on the blood flow and radiation response of solid murine tumors.  $^{133}\text{Xe}$  clearance was used to measure KHT tumor blood flow following pentobarbital, urethane, nitroglycerin, and metaraminol administration *in vivo*. Clonogenic cell survival was measured to relate tumor blood flow alterations with changes in the hypoxic cell fraction.

## METHODS AND MATERIALS

### Tumors

The tumor used in these studies was the KHT sarcoma, a transplantable tumor of  $\text{C}_3\text{H}$  mice.<sup>13</sup> It was transplanted by intramuscular injection of  $4 \times 10^5$  cells into the left hind leg of 8-14 week old  $\text{C}_3\text{H}/\text{HeDub}$  male mice (22-27 g in weight). Experiments were performed after nine days when the tumors had grown to a diameter of approximately 10-11 mm (0.6-0.7 g).

### Blood flow measurements

Tumor blood flow was measured using a modification<sup>18</sup> of the  $^{133}\text{Xe}$  ( $^{133}\text{Xe}$ ) clearance technique described by Kallman *et al.*<sup>12</sup> The mouse was positioned in the same jig utilized for irradiation, and placed in an acrylic box with a 22 mm diameter aperture in a lead shield above and below the tumor-bearing leg. A small volume (20-30  $\mu\text{l}$ ) of  $^{133}\text{Xe}$  dissolved in saline was injected into two sites on opposite sides of the tumor and the clearance of radioactivity was monitored as a function of time after injection, using a Picker Dyna Camera 2C with a NaI crystal. Blood flow was calculated in ml/100g/min using the partition coefficient which was previously obtained for this tumor.<sup>18</sup> Repeated tumor blood flow measurements indicate good reproducibility of the experimental tech-

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nique as demonstrated by the results presented in Figure 1.

### Treatments

The effect of anesthetics on tumor blood flow was determined in mice anesthetized by an intraperitoneal injection of either sodium pentobarbital\* (70 mg/kg) or urethane (1.2 mg/kg). Blood flow was determined prior to and then 8–10 minutes after administration of the anesthetic.

Nitroglycerin† (10 mg/mouse) was dissolved in saline immediately before subcutaneous injection and blood flow was determined 15 and 20 min thereafter. Metaraminol bitartrate‡ (1 mg/mouse) was injected intraperitoneally and blood flow determined 8–10 min later.

### Irradiations and assay system

Nonanesthetized or anesthetized/drug-treated animals were confined as previously described<sup>17</sup> and the tumor-bearing leg was locally irradiated with 2000 rad by a double-headed 100 KVp X ray unit at a dose rate of 1140 rad/min. The survival of cells in the KHT tumor immediately after irradiation was determined using an *in vitro* agar colony assay.<sup>26</sup>

### Temperature measurements

Tumor temperature was measured using an implantable thermocouple in a 29 gauge needle (Type MT-29/1) and rectal temperature was monitored using a teflon-coated thermocouple (diameter 0.64 mm—Type IT-18) inserted deep into the rectum. Both of these thermocouples were connected to a digital thermometer§ to give readings with an accuracy of  $\pm 0.1^\circ\text{C}$ . Measurements were made both on non-anesthetized mice and on mice anesthetized with pentobarbital and urethane. The measurements were made under the same conditions used in the blood flow determinations and radiation exposure.

## RESULTS

The effects of vasoactive agents and anesthetics on tumor blood flow are shown in Table 1. The blood flow of each tumor was measured prior to drug treatment and at a specified interval following drug administration. Nitroglycerin produced a small but insignificant decrease in tumor blood flow at either 15 or 30 min after injection. Metaraminol reduced tumor blood flow to an average of 60% of the pretreatment value.

Pentobarbital and urethane reduced blood flow to approximately 41 and 76% of the pretreatment values, respectively.

Tumor radiation response following treatment of tumor-bearing animals with urethane, pentobarbital, and metaraminol was measured using the *in vitro* cell survival assay. Animals received either pentobarbital or urethane

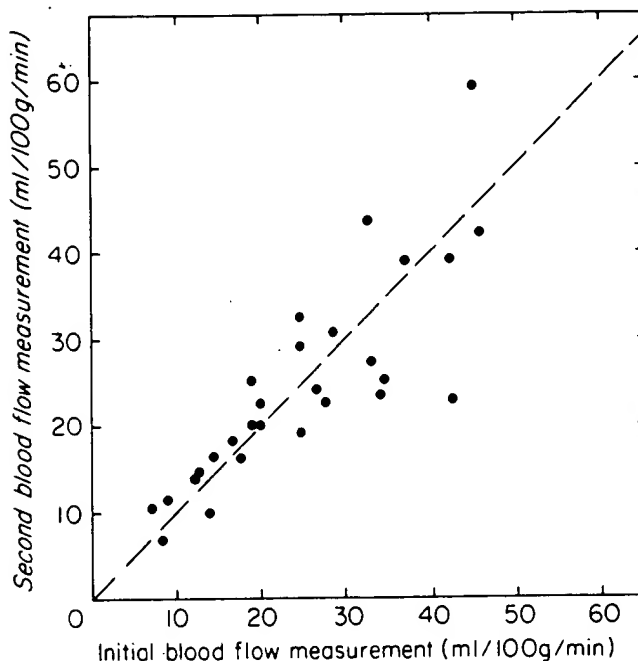


Fig. 1. Two separate blood flow measurements were made on each of a series of tumors with the second measurement being made 1.5–24 hr after the initial measurement. The second measurement is plotted relative to the initial measurement with each point being for a different tumor. The broken line indicates where the two measurements would be identical.

and 8 min later were irradiated with 2000 rad. Metaraminol-treated mice were irradiated 10 min after drug injection. Tumor cell survival measurements, shown in Table 2, indicate that both the anesthetic treatments and metaraminol exposure increased the surviving fraction approximately three-fold.

We measured tumor and rectal temperatures at 10–100 min following anesthetic treatment of groups of 2–4 mice. These data are shown in Figure 2. Pentobarbital produced a pronounced and prolonged temperature

Table 1. Effects of vasoactive agents on tumor blood flow

N	Agent	Initial blood flow ml/100 g/min	Blood flow ratio
12	Nitroglycerin (15 min)	$23.1 \pm 2.7$	$0.88 \pm 0.20$
12	Nitroglycerin (30 min)	$20.5 \pm 2.1$	$0.88 \pm 0.13$
7	Metaraminol	$25.5 \pm 0.93$	$0.60 \pm 0.11$
23	Urethane	$25.3 \pm 1.6$	$0.76 \pm 0.06$
24	Pentobarbital	$27.0 \pm 1.9$	$0.41 \pm 0.06$

Note: Values represent the mean ( $\pm$  S.E.) blood flow of (N) of KHT sarcomas per group. Blood flow values were made 8–10 min after drug injection, (except as noted).

Blood flow ratio = Post-treatment blood flow/Initial blood flow.

\*Nembutal, Abbott.

†Parke-Davis.

‡Aramine, Merck, Sharp & Dohme.

§Model BAT-8C, Bailey Instruments, Saddle Brook, NJ.

Table 2. Effect of vasoactive agents on tumor cell survival

Agent	N	Surviving fraction ( $\times 10^3$ )	
		Control	Treated
Pentobarbital	4	1.8 (2.8 - 1.2)	6.6 (8.4 - 5.2)
Urethane	4	1.8 (2.8 - 1.2)	5.8 (6.4 - 5.3)
Metaraminol	4	1.0 (1.9 - 0.5)	2.6 (3.3 - 2.0)

Note: Values represent the mean (S.E. range) of N independent survival determinations. Drug doses as listed in Table 1. The KHT tumor was irradiated *in vivo* with 2000 rad. Control values are the surviving fraction of tumors which received irradiation alone.

decrease in both the whole body ( $10^\circ\text{C}$ ) and the tumor ( $8^\circ\text{C}$ ). Urethane produced a maximum reduction of  $1-2^\circ\text{C}$  in both tumor and core temperatures, which was regained as the animal recovered from the anesthetic at 30-40 min after injection. Simple immobilization of the animal in the jig also resulted in a drop in tumor temperature of  $1-1.5^\circ\text{C}$  and a smaller drop in rectal temperature (see Fig. 2b and c). Figure 2c indicates that probe position in the tumor may significantly affect temperature readings, hence a single temperature measurement such as was done in Fig. 2a and b is unlikely to be representative of the whole tumor. However, similar patterns of temperature changes were obtained with both temperature probes.

### DISCUSSION

We measured tumor blood flow using a radioactive  $^{133}\text{Xe}$  clearance technique which we described earlier.<sup>18</sup> The validity of the technique depends upon the rapid diffusion of  $^{133}\text{Xe}$  molecules from the tissue to the blood stream where they are carried to the lungs and expired. Blood flow measurements obtained by this procedure are believed to represent arterial capillary blood flow.<sup>1</sup> The  $^{133}\text{Xe}$  clearance procedure is not cytotoxic and enables one to make consecutive measurements of tumor blood flow in a highly reproducible manner. Thus, the effects of a particular treatment on the blood flow of an individual tumor can easily be assessed, as well as those on clonogenic cell survival.

In the KHT tumor, blood flow was reduced 24 and 59% at 8-10 min after treatment with urethane and pentobarbital, respectively. Kallman *et al.*<sup>12</sup> reported very similar results for the KHT tumor using pentobarbital, and blood flow in a number of other mouse tumors has also been shown to decrease following treatment with pentobarbital or urethane.<sup>4,10,12,28</sup> A large pentobarbital-induced decrease in core and tumor temperature has been reported by Rockwell and Loomis,<sup>20</sup> Sheldon *et al.*<sup>22</sup> and Johnson *et al.*<sup>10</sup> Urethane generally seems to produce smaller temperature decreases,<sup>4,10</sup> similar to the results presented here. These results of our study suggest that there is a general relationship between tumor blood flow reduction and decreased tumor and core temperatures.

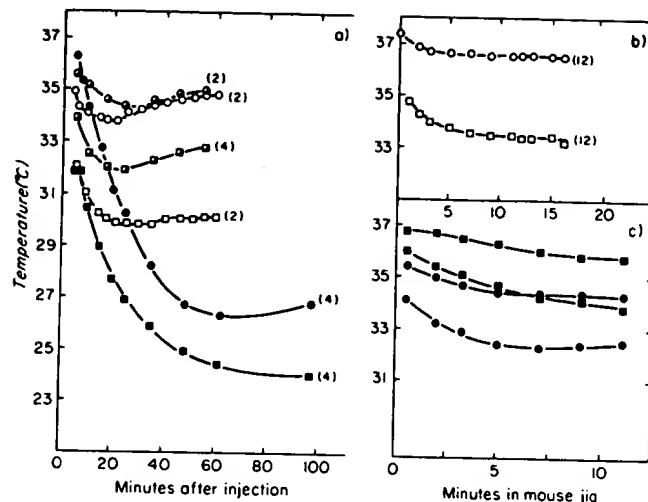


Fig. 2. (a) Tumor (■, □, ○) and rectal (●, ●, ○) temperatures plotted as a function of time after injection of the host animal with pentobarbital (■, ●) or urethane (□, □, ○, ○). The points are mean values of measurements obtained on the number of animals indicated in parenthesis beside each curve. A single temperature probe was used for the tumor temperature measurements. (b) Tumor (□) and rectal (○) temperatures in non-anesthetized mice held in the treatment jig. Mean values for a group of 12 mice. (c) Individual temperature measurements made on two tumors in non-anesthetized mice, where there were two probes in each tumor. The different symbols represent the different tumors.

Although both core and tumor temperature of untreated animals declined slightly as a function of time in the irradiation jig, there was no detectable change in blood flow under these conditions.<sup>18</sup>

The present results demonstrate that pretreatment with either urethane or pentobarbital results in a three-fold increase in tumor cell survival following radiation. Since the well-oxygenated tumor cells are killed at this radiation dose (2000 rad),<sup>7</sup> it is likely that the increase in tumor cell survival reflects a change in the apparent properties of hypoxic cells. The hypoxic fraction increased three-fold following both anesthetics; however, the magnitude of this increase was not directly correlated with the 24% (urethane) and 59% (pentobarbital) decrease in blood flow, or the  $2^\circ$  and  $10^\circ$  decrease in tumor temperature observed following urethane and pentobarbital, respectively. Since the apparent hypoxic fraction of KHT tumors in non-anesthetized mice is quite large,<sup>7</sup> it is possible that both anesthetics increased it to the maximum extent, e.g., under anesthesia the tumor is responding as if virtually all the cells are hypoxic. The survival levels ( $3-6 \times 10^{-3}$ ) following anesthesia are approximately equivalent to those of a completely hypoxic tumor cell population.<sup>7</sup> The hypothesis of an upper limit to the proportion of hypoxic cells is supported by data of Rockwell and Loomis<sup>20</sup> which showed that anesthesia has no effect on radiation response of anoxic tumors.

Administration of vasoactive agents to tumor-bearing animals appears to affect tumor blood flow and vascular

resistance to varying degrees.<sup>2,5,6,9,14,16,19,25,27</sup> Our most consistent blood flow perturbations were obtained with metaraminol, a sympathomimetic agent whose overall effects on normal tissue are similar to those of norepinephrine, although the former drug is less potent and has a longer duration of action.<sup>8</sup> The 40% reduction in tumor blood flow which we observed following metaraminol treatment may be due either to a direct action of metaraminol on tumor vasculature, or result indirectly from peripheral vasoconstriction. The apparent tumor hypoxic cell fraction was increased two to three-fold following metaraminol treatment.

The present study was undertaken to better understand

the relationship between tumor blood flow and acutely-induced changes in tumor radiosensitivity. Treatment with anesthetics and metaraminol reduced blood flow and increased the fraction of hypoxic cells in the tumor. However, the magnitude of these changes was not linearly related, suggesting that factors (e.g., cell proliferative status), other than blood flow, contribute to tumor radiosensitivity. The data indicate that an overall reduction in blood flow can be expected to decrease tumor radiosensitivity. Recently there have been reports of drugs which preferentially increase tumor blood flow.<sup>11,24</sup> It would be very interesting to determine if such drugs can increase tumor radiosensitivity.

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# The Hypoxic Cell: A Target for Selective Cancer Therapy—Eighteenth Bruce F. Cain Memorial Award Lecture<sup>1</sup>

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## Abstract

It has been appreciated for more than 50 years that very low levels of oxygenation, or hypoxia, both protect cells from killing by X-irradiation and are present in solid tumors but not in normal tissues. Until recently, however, there has been no definitive proof that hypoxia in human tumors contributes to radiotherapy treatment failure. We now know that hypoxia in solid tumors is not only a major problem for radiation therapy but also leads to resistance to most anticancer drugs and, importantly, appears to accelerate malignant progression and increase metastasis. To date, efforts to overcome the problem of hypoxia have had only limited success. However, the recent development of new drugs that are nontoxic until they are activated in the hypoxic cell opens a new era. The first of these new drugs to be tested clinically, tirapazamine, a drug that is highly toxic to hypoxic but not aerobic cells, has already demonstrated efficacy in selective potentiation of cisplatin in randomized Phase III trials with non-small cell lung cancer. The unique presence of hypoxic cells in human tumors provides an important target for selective cancer therapy.

## Introduction

Research on methods of overcoming the problem for radiotherapy of hypoxic cells in solid tumors has been ongoing for almost 50 years. During that time, interest among basic researchers has waxed and waned as promising new directions emerged, only to fail in clinical trials. However, as increasingly sophisticated concepts have replaced earlier, simpler ideas, the prospect of overcoming and eventually exploiting this fundamental difference between normal and malignant tissues appears more realistic. Recent clinical studies have for the first time unequivocally demonstrated that hypoxia in solid tumors is a major problem for radiotherapy, and that low oxygenation can accelerate malignant progression and metastasis, thereby creating a poorer prognosis irrespective of which cancer treatment is used. Development of new drugs that are selectively toxic to hypoxic cells, of which TPZ<sup>3</sup> is a prototype, has a solid theoretical and preclinical base and is also showing positive clinical results. This review will trace some of the important concepts and developments that have brought us to the present in this field.

## The Problem for Radiotherapy of Hypoxia in Solid Tumors

Although it had been appreciated for several years that lowering the oxygenation of tissues made them more resistant to damage by ionizing radiation (1), it was the pioneering studies of Gray and colleagues soon after World War II that established the universality of the radiation resistance conferred by hypoxia as well as providing early insight into the

mechanism of action. In their landmark paper, Gray *et al.* (2) showed that hypoxia conferred resistance to radiation damage of a wide range of cells and tissues using various end points. They showed further that it was the presence of oxygen at the time of irradiation that caused radiation sensitivity rather than any metabolic effects, as had been previously supposed. The oxygen effect may be unique in biology in having such a broad applicability: it applies to enzymes in solution; to bacteria; to yeast; and to plant and mammalian cells irrespective of their genetic background. A typical radiation killing curve for mammalian cells under aerobic and hypoxic conditions is shown in Fig. 1. The difference in radiation sensitivity between the aerobic and hypoxic cells, which is known as the oxygen enhancement ratio and is defined as the ratio of doses to produce the same level of cell kill under hypoxic to aerobic conditions, is normally in the range 2.5–3 for mammalian cells. Although this ratio may not seem large, the difference in cell kill for a given radiation dose can be several orders of magnitude, as can be seen in Fig. 1 for a dose of 14 Gy (*dashed vertical line*). The reason for the universality of this effect is that oxygen reacts chemically with the fundamental biological lesion produced by ionizing radiation, a radical in DNA. Oxygen, being the most electron-affinic molecule in the cell, reacts extremely rapidly with the free electron of the free radical, thereby “fixing” (making permanent) the damage. In the absence of oxygen, much of the radical damage can be restored to its undamaged form by hydrogen donation from nonprotein sulfhydryls in the cells. This mechanism is shown in Fig. 1. Thus, ionizing radiation is severely compromised in its ability to kill hypoxic cells. This is true for all cell types, both normal and malignant. However, the degree of resistance can be changed somewhat by the nature of the radiation. Very densely ionizing radiation such as  $\alpha$  particles shows no effect of cellular oxygenation on their killing ability, with particles of intermediate ionization densities such as fast neutrons having an intermediate effect (3). This has been the motivation for developing neutron beams for therapy, but the expense and other problems of such modalities have prevented their widespread acceptance. For the foreseeable future, the vast majority of radiotherapy will be conducted with X-rays.

Insight into how hypoxia can develop in tumors was provided by Thomlinson and Gray (4), who showed with histological sections of human tumors that there was a constant distance across tumor tissue between blood vessels and necrosis. Furthermore, they showed that this distance (usually 100–150  $\mu$ m) was predicted as the oxygen diffusion distance, given the oxygen partial pressure in capillaries and the rate of oxygen consumption of cells. They suggested that viable hypoxic (and hence radioresistant) cells would be adjacent to the necrotic areas in tumors. This theory has stood the test of time. There are now several different methods of visualizing hypoxic cells in sections of rodent and human tumors (5–7), and these have generally shown hypoxia at the predicted distances from blood vessels and also adjacent to necrosis if present.

Of the techniques presently available for detecting hypoxia in human tumors, the most quantitative and widely used is a commercially available, polarographic oxygen electrode ( $pO_2$  histogram; Eppendorf, Hamburg, Germany). The sensing electrode, mounted on

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<sup>3</sup> The abbreviations used are: TPZ, tirapazamine; HCR, hypoxic cytotoxicity ratio.

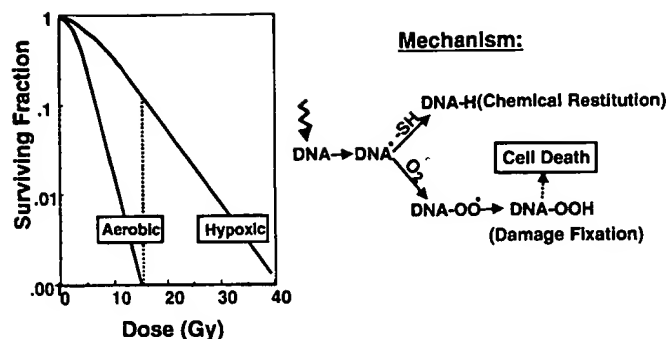


Fig. 1. Typical survival curves to ionizing radiation for mammalian cells under aerobic and hypoxic conditions. Most mammalian cells, irrespective of genetic background, exhibit a survival curve with an initial "shoulder" region followed by exponential cell killing. The oxygen enhancement ratio (ratio of doses to produce the same cell kill under hypoxic to aerobic conditions) is typically 2.5–3.0 and, in this figure, is 2.8. The dotted vertical line at 14 Gy shows the >2 logs difference in cell kill for aerobic and hypoxic cells at this dose. Also shown is the mechanism for the greater sensitivity of aerobic cells as compared to hypoxic cells. Ionizing radiation produces a radical in DNA, which can be either chemically restituted by donation of hydrogen from nonprotein sulfhydryls ( $-SH$ ) in the cell or, in the presence of oxygen, converted into permanent damage that increases the probability of cell death.

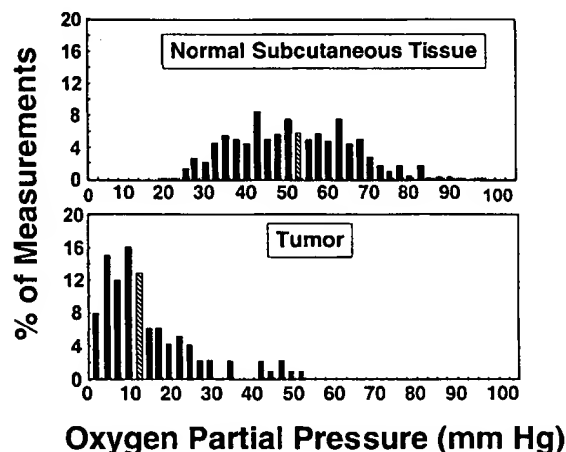


Fig. 2. Oxygen distribution in a lymph node metastasis of a head and neck tumor and in the surrounding normal s.c. tissue. These measurements were made using the Eppendorf oxygen electrode in a single patient in a series reported by Adam *et al.* (16). ■, the median value for the measurement.

the tip of a stiff needle, is advanced automatically through the tissue, taking readings sufficiently rapidly (within 1.4 s) to avoid changes in oxygen tension resulting from pressure artifacts or tissue damage caused by the needle. Using this system, a histogram of oxygen tensions can be obtained from multiple points along different tracks through the tissue. An example of measurements made in a neck node of a squamous cell carcinoma and adjacent normal tissue is shown in Fig. 2. The normal tissue shows a typical Gaussian distribution of oxygen tensions with a median between 40 and 60 mm Hg, and no values less than 10 mm Hg. Tumors, on the other hand, invariably show a distribution with much lower oxygen tension. The one shown in Fig. 2 is typical in the sense that in many series of human tumors at different sites, roughly half the tumors have a median value of less than 10 mm Hg, and half have a median greater than this (8–12). This median of 10 mm Hg is also significant in that this is the point at which radiation resistance starts to develop with full resistance at values of less than approximately 0.5 mm Hg. Thus, the tumor shown in Fig. 2 would be expected to have regions with cells at greater radiation resistance than those in adjacent normal tissues.

The availability of this technology has led to investigators asking the question of whether the more hypoxic tumors had an inferior

outcome to radiotherapy. This has now been demonstrated by a number of studies both with head and neck tumors and with carcinomas of the cervix (12–16). Fig. 3 shows data from one of these studies, demonstrating a large effect of tumor oxygenation on outcome, despite the fact that there was no difference in any other prognostic factor between the two groups. In general, this has been the finding of several investigators: tumor hypoxia does not depend on tumor size, grade, extent of necrosis, or patient hemoglobin levels, and is therefore an independent predictor of outcome.

Thus, the conclusions reached by the pioneering studies of Gray, Thomlinson and colleagues were correct: hypoxic cells *are* present in human solid tumors, and they *do* negatively influence the outcome of radiotherapy. However, they were only partially correct. Tumor hypoxia has turned out to be both more complicated and of greater significance than they envisaged.

### Tumor Hypoxia Is Also a Problem for Chemotherapy

Hypoxic cells *in vitro* are universally resistant to ionizing radiation, but not to anticancer drugs (17, 18). Exceptions are bleomycin and neocarzinostatin, which, like radiation, are more toxic toward oxygenated cells, and bioreductive drugs that are more toxic toward hypoxic cells. However, in a solid tumor *in vivo*, a number of factors associated either directly or indirectly with tumor hypoxia contribute to resistance to anticancer drugs.

First, hypoxia (and possibly hypoxia-associated deficiencies in other nutrients such as glucose) causes cells to stop or slow their rate of progression through the cell cycle (19, 20). This effect is not the result of a generalized decrease in ATP or energy status of the cell but is likely to be caused by specific proteins induced under hypoxic conditions (21–24). Because most anticancer drugs are more effective against rapidly proliferating cells than slowly or nonproliferating cells, this slowing of cell proliferation with increasing distance from the vasculature will lead to decreased cell killing at these increased distances.

Second, the concentration of anticancer drugs will be higher closer to blood vessels than further away. This is a consequence not only of the geometry, in which the drug being provided by a central vessel has to diffuse out over a much greater volume at the periphery of the cord, but also of the fact that many anticancer drugs, because of their reactivity, will be limited in their diffusion from the blood vessel. This is particularly true for agents that physically bind to DNA, such as intercalators (25–28). Even for non-DNA intercalators, such as melphalan, there can be a problem in nonhomogeneous distribution from the blood vessel (29).

There are also other ways in which hypoxia might contribute to drug resistance. One is through amplification of genes conferring drug resist-

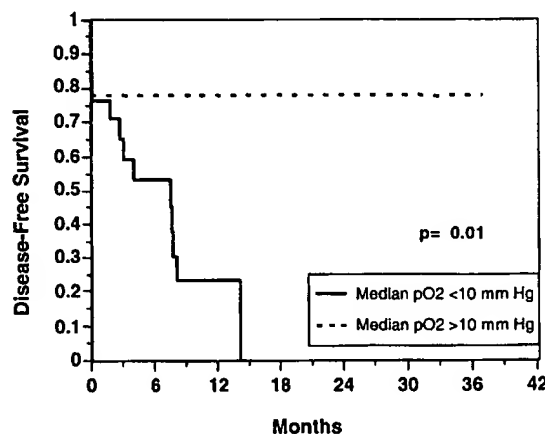


Fig. 3. Data from Brizel *et al.* (12) showing the influence of median oxygenation of head and neck tumors on disease-free survival after standard radiotherapy.



ance (30) or through the induction of various hypoxic stress proteins that appear to be responsible for resistance to Adriamycin (31), etoposide (32), and cisplatin (33). Another factor that can affect response to chemotherapy is extracellular pH, which is likely to be lower in the hypoxic areas of the tumor than in the well-oxygenated areas. Because the intracellular pH of the cell is highly regulated, it will not fall as low as the extracellular pH levels, thereby creating a pH gradient with more acidic levels outside the cell. Such *trans*-membrane pH gradients inhibit the intracellular accumulation of weak bases, such as Adriamycin (34), although the reverse is true for weak acids such as chlorambucil (35).

In summary, there are a number of well-established phenomena that will cause a gradient of reduced cell killing by most anticancer agents as a function of distance from the vasculature (Fig. 4). Such a gradient has been shown in experimental tumors and in spheroids (27, 36).

#### Tumor Hypoxia Increases Malignant Progression and Metastasis

Recent studies have shown that hypoxia in solid tumors has an important consequence in addition to conferring a direct resistance to radiation and chemotherapy. Graeber *et al.* (37) have demonstrated that low oxygen levels cause apoptosis in minimally transformed mouse embryo fibroblasts, and that this apoptosis depends to a large extent on wild-type p53. Using these same cells growing as solid tumors in immune-deprived mice, they further showed that apoptosis colocalizes with hypoxic regions in tumors derived from p53 wild-type mice. In tumors derived from p53<sup>-/-</sup> cells, there was much less apoptosis and no colocalization with tumor hypoxia. These findings provide evidence that hypoxia, by selecting for mutant p53, might predispose tumors to a more malignant phenotype. In addition to this selection of cells with mutant p53 (hence, cells more likely to mutate), hypoxia and the tumor microenvironment have also been shown to cause mutations directly (38, 39). Clinical data support this conclusion: studies with soft tissue sarcomas (40) and with carcinoma of the cervix (41, 42) have shown that hypoxia is an independent and highly significant prognostic factor predisposing tumors to metastatic spread.

Tumor hypoxia also stimulates tumor progression by promoting angiogenesis through the induction of proangiogenic proteins such as vascular endothelial growth factor (43). Most genes induced by hypoxia are regulated by the hypoxia-inducible transcription factor HIF-1, a protein that therefore plays a very important role in tumor development (44–46).

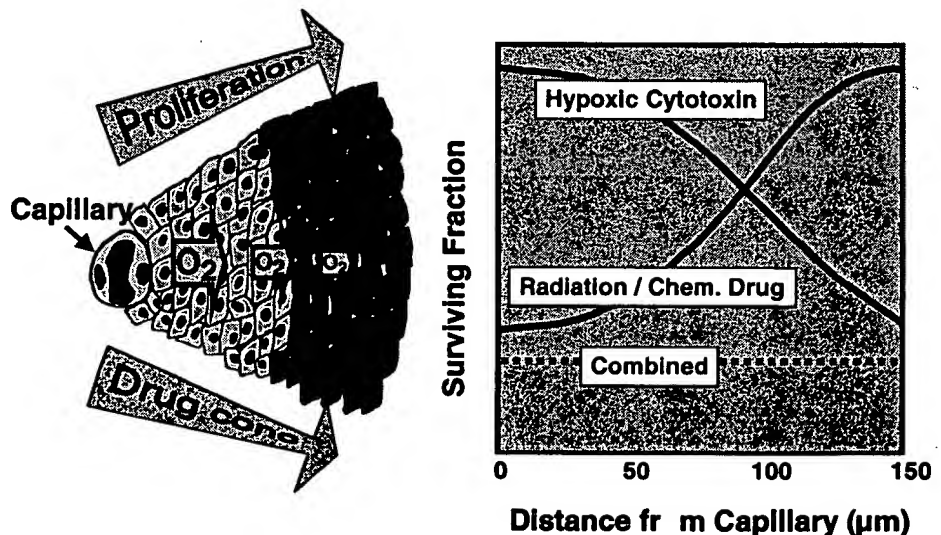
#### Fluctuating Blood Flow in Tumors

Despite the strong evidence for the Thomlinson-Gray model of development of hypoxia in tumors, a number of observations began to develop in the late 1970s that did not seem to fit the model. One was from our own experiments with the hypoxic radiosensitizer misonidazole, which, when given at very high doses to tumor-bearing mice, produced extensive tumor cell kill that was located around regions of necrosis (47). According to the classic model, this should have eliminated or markedly reduced the number of viable hypoxic cells in the tumor. However, when we measured the fraction of radiation-resistant (and therefore, hypoxic) cells in the tumor, we saw little change (48). Also, in carefully performed experiments with tumors growing between two transparent windows, Yamaura and Matsuzawa (49) found that after a large, fixed dose of radiation, tumors regrew from regions that were highly vascularized rather than (as expected) areas adjacent to necrosis. Although these authors did not invoke acute or fluctuating hypoxia as an explanation for this, it seemed to us that this was a hypothesis that could account for both our own data and their data. We therefore suggested that blood flow in tumors could fluctuate, thereby producing temporary regions of acute hypoxia (48).

Later studies using vascular casting techniques with window chamber preparations identified the structural basis for inhomogeneities in blood flow in transplanted tumors (50–52). These investigations showed that tumor blood vessels are tortuous, highly irregular, have arterial venous shunts, blind ends, and lack smooth muscles and innervation. As a result, blood flow would be expected to be sluggish and highly irregular. At about the same time as these anatomical studies, fluctuating blood flow in transplanted tumors was convincingly demonstrated using two diffusible dyes injected i.v., either together or several minutes apart (53–55). Essentially, these investigators found that in tumor sections from mice injected with the two dyes several minutes apart, a fraction of the blood vessels was labeled with one dye or the other (not both), implying vessel opening or closing in the time interval between the two injections.

One of the important implications of fluctuating blood flow is that not only would the hypoxic cells produced by temporary cessation of blood flow be resistant to ionizing radiation, but it is also likely that they would be resistant (because of inadequate drug delivery) to any anticancer drug that had a half-life in the blood stream that was less than or equal to the time for which the blood vessel remained closed. Thus, as in the case of

Fig. 4. Left, a diagrammatical representation of part of a tumor cord surrounding a capillary showing decreasing oxygen concentration as well as decreasing cellular proliferation and drug concentration as a function of distance from the capillary. Right, the considerations on the left lead to the prediction that cell killing by radiation or most anticancer drugs will be reduced as a function of distance from the capillary. On the other hand, a drug with a high preferential toxicity for hypoxic cells (a hypoxic cytotoxin) should show the opposite profile. The combination of standard treatment with such a hypoxic cytotoxin would be expected to overcome the problem of hypoxic cells by producing a relatively uniform cell profile of cell killing as a function of distance from the capillary (*Combined*). Such profiles have been demonstrated experimentally for human tumors transplanted into mice by Durand (36).



chronic hypoxia, acutely hypoxic cells would be expected to be resistant to both radiation and commonly used anticancer drugs.

Are both of these types of hypoxia, acute and chronic, present in human tumors? Several investigators have clearly demonstrated the presence of chronically hypoxic cells using hypoxia-activated markers (7).<sup>4</sup> Acute hypoxia cannot be visualized directly in human tumors, but studies by Hill *et al.* (56) of tumor blood flow using laser Doppler probes have shown that fluctuating blood flow occurs in human tumors in a manner similar to that in mouse tumors. Thus, we can be reasonably confident that both of these types of hypoxia are common in human tumors.

### Hypoxic Cell Radiosensitizers: A Good Idea That Might Have Worked

One of the earliest attempts to overcome the problem of the resistance of hypoxic cells in tumors to radiotherapy was to increase oxygen levels in the blood stream, thereby increasing the diffusion distance of oxygen. A number of trials were performed with patients breathing 100% oxygen at a pressure of 3 atmospheres, but the results were mixed (57–59). In retrospect, we can now appreciate that this strategy would only reduce the number of chronically hypoxic cells; it would not be expected to change the proportion of acutely hypoxic cells.

As interest waned in the use of hyperbaric oxygen, an apparently superior solution seemed at hand. Following pioneering studies by Adams *et al.* (60, 61) on the use of electron-affinic drugs to sensitize hypoxic bacteria and mammalian cells *in vitro*, a drug was identified as showing activity with transplanted murine tumors (62, 63). This drug, a 5-nitroimidazole already used clinically as Flagel, showed excellent activity in sensitizing mouse tumors to radiation. Almost immediately a more active drug, a 2-nitroimidazole, later named misonidazole, became available (64). The results obtained for these drugs were quite remarkable: with large, single doses of radiation, large degrees of sensitization of tumors with no concomitant sensitization of normal tissues were found by a number of groups (64–66). Clinical trials were started almost immediately, but a major side effect with multiple doses emerged that had not been seen in preclinical studies: misonidazole produced a severe neuropathy, both peripheral and central, that limited the dose of drug that could be used with radiotherapy (67). Not surprisingly, almost all of the clinical trials of radiotherapy combined with misonidazole turned out to be negative (68), an outcome consistent with the small degree of radiosensitization expected with the clinically used doses (69). It should be noted, however, that a more recent meta-analysis of all of these trials has demonstrated a small but significant benefit of misonidazole and other hypoxic radiosensitizers when added to radiotherapy (70). At the time, however, the problem of neurotoxicity seemed to be an overwhelming limitation to the success of misonidazole.

But could the neurotoxicity of these drugs be eliminated while retaining their radiosensitizing properties? It seemed to us that this was a possibility. Because the mechanism of hypoxic radiosensitizers is similar to that of oxygen in that they fix the free radical damage to DNA, the extent of their radiosensitization depends on the concentration of drug at the time of irradiation. Drug toxicity, on the other hand, is likely to depend on area under the concentration  $\times$  time curve for specific tissues. This is different from anticancer drugs whose efficacy and toxicity are both dependent on area under the concentration  $\times$  time curve. This feature of radiosensitizers made it attractive to try to design drugs rationally to minimize tissue exposure, particularly to neural tissues. William Lee, who led a chemistry group at SRI International, and my laboratory at Stanford University set about to do just this. Our strategy was based on the simple concept illustrated in Fig. 5. Consistent with this concept, we

found that as we decreased the lipophilicity of the compounds while keeping the 2-nitroimidazole moiety that had been shown to determine radiosensitivity, we were able to reduce drug exposure to the brain and to reduce toxicity (71, 72). Eventually, however, at too great a hydrophilicity, the drugs failed to enter the tumor cells and hence lost antitumor efficacy. The optimum drug, SR2508, later known as etanidazole, was three to four times less toxic to mice but had the same antitumor efficacy (73). Subsequent clinical trials confirmed that three times more etanidazole than misonidazole could be given without producing neurotoxicity (74). However, a recently reported Phase III randomized trial of radiotherapy of head and neck cancer with or without etanidazole has failed to show a significant overall benefit of adding the drug (75).

Why was this drug not more effective? In the first place, giving three times more drug only increased the expected radiosensitization of the hypoxic cells from an enhancement ratio of 1.1–1.2 to approximately 1.4. However, a more serious problem is that it is not the cells at maximum radiation resistance but those at intermediate oxygenation and intermediate radioresistance that dominate the response to fractionated irradiation. This has been demonstrated in a recent theoretical analysis of the influence of hypoxia on radiotherapy and is likely to be a problem with any hypoxic cell radiosensitizer (76). Unfortunately, although hypoxic cell radiosensitizers at clinically realistic doses can sensitize the maximally hypoxic cells to radiation killing, they have little effect on the radiosensitivity of the cells at intermediate hypoxia and radiosensitivity. This is a consequence of the logarithmic nature of the curve of radiosensitization *versus* dose; as cell sensitivity increases, it takes geometrically more drug to increase their radiosensitivity still further. Thus, a hypoxic cell partially sensitized by some oxygen takes much more drug to increase its sensitivity by a given amount compared to a fully hypoxic cell. It thus seems unlikely that hypoxic cell radiosensitizers, even newer ones with greater potency, will influence the outcome of fractionated radiotherapy. However, they may and probably should play a role in those situations where large, single doses of radiation are given, such as with stereotactic radiotherapy of brain tumors.

### Hypoxic Cytotoxins: Turning Hypoxia from Problem to Advantage

Can the low oxygen levels in tumors be turned into a therapeutic advantage? Such a possibility was proposed over 25 years ago by Lin *et al.* (77), who reasoned that compounds based on the quinone structure of mitomycin C might be more active in hypoxic tumors. It was known at that time that mitomycin C required metabolic reduction of the benzoquinone ring to produce the cytotoxic bifunctional alkylating agent. Lin *et al.* reasoned that a lower oxidation reduction (redox) potential for tumor tissue relative to most normal tissues (which had been suggested by earlier work of Cater and Philips; Ref. 78) could increase reductive activation of these quinone derivatives in tumors. Although this was not the correct mechanism for the increased

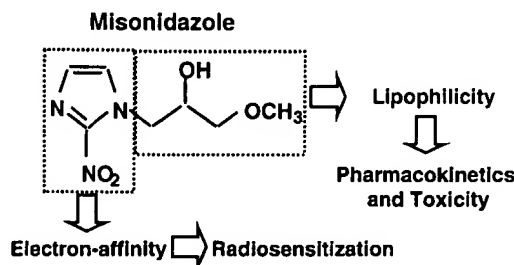


Fig. 5. The rationale for producing a less toxic hypoxic cell radiosensitizer than misonidazole was based on changing the side chain of the drug so as to reduce lipophilicity, thereby increasing the drug clearance rate and inhibiting its access to neural tissues. This figure was adapted from Ref. 72.

<sup>4</sup> S. M. Evans and C. J. Koch, personal communication.

cytotoxicity of mitomycin C and certain analogues toward hypoxic cells (much lower levels of hypoxia are needed to change cellular redox potential), these studies were important in suggesting the potential of hypoxia-activated drugs and led to the concept of selectively killing the hypoxic cells in solid tumors (79–83).

It is important to note that specifically killing the hypoxic cells in tumors has greater therapeutic potential than oxygenating the cells or chemically sensitizing them to radiation or chemotherapy for two reasons: (a) hypoxic cytotoxins specifically kill those cells resistant to radiation and chemotherapy, thereby producing complementary cytotoxicity as shown in Fig. 4; and (b) the random fluctuations in acute hypoxia that are thought to occur in tumors create a situation in which hypoxia could be a therapeutic advantage. Modeling studies, the results of which are shown in Fig. 6, illustrate that if a hypoxic cytotoxin can be given with every radiation dose in a radiotherapy regime, then the overall cell kill in a hypoxic tumor can be greater than if the tumor were fully oxygenated. This occurs when the hypoxic cytotoxin kills 50% or more of the hypoxic cells each time it is given. It is important to note that this requirement of giving the hypoxic cytotoxin with many, if not all, of the radiation doses would be difficult to achieve with a conventional chemotherapy drug that kills aerobic cells. Thus, although randomized clinical trials have demonstrated that the addition of the bioreductive drug mitomycin C to radiotherapy has shown a clear benefit (84, 85), the fact that it could only be given two times during the course of radiotherapy makes it unlikely that the drug was exploiting fluctuating hypoxia. Nonetheless, these results with mitomycin C are clearly promising for the use of hypoxia-activated agents with radiotherapy.

#### TPZ: A Novel Hypoxic Cell Toxin

Serendipity played a large role in the discovery of the benzotriazine di-*N*-oxide class of hypoxic cytotoxins of which TPZ is the lead compound. In the mid-1980s, William Lee and I were looking for new classes of hypoxic radiosensitizers that did not include a nitro group, which we surmised might be responsible for the neurotoxicity of the 2-nitroimidazole class of radiation sensitizers. In testing a particular benzotriazine di-*N*-oxide, SR 4233, (later known as TPZ), we found not only that it killed hypoxic cells at much lower concentrations than those needed to radiosensitize the cells, but also that its differential toxicity to hypoxic cells was much larger than that of any known drug. The HCR, or the concentration of drug required under aerobic relative to hypoxic condi-

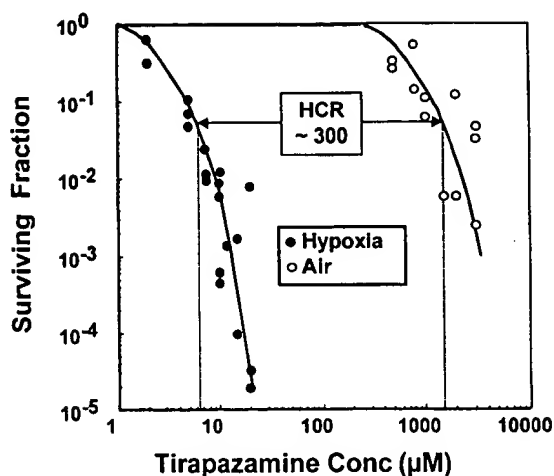


Fig. 7. Killing of hypoxic and aerobic mouse SCCVII tumor cells assayed by clonogenic survival after a 1-h exposure of the cells to TPZ under either hypoxic or aerobic conditions *in vitro*. The HCR is the ratio of drug concentrations under aerobic:hypoxic conditions to produce equal cell kill; in this tumor cell line, the HCR is approximately 300. This figure was adapted from Ref. 106.

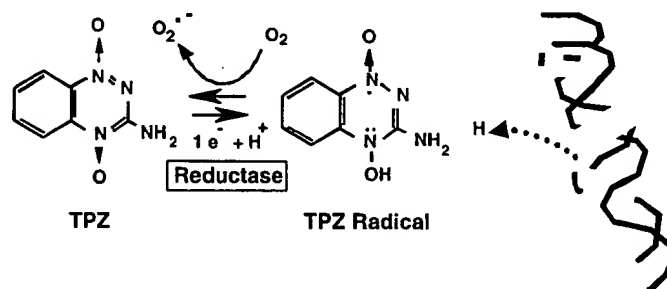


Fig. 8. The mechanism of the preferential hypoxic cytotoxicity of TPZ. Under both aerobic and hypoxic conditions, the drug is reduced by an intracellular reductase to form a highly reactive radical, which can cause DNA single- and double-strand breaks. In the presence of oxygen, however, oxygen will take the free electron to itself, thereby back-oxidizing the radical to the inactive parent drug.

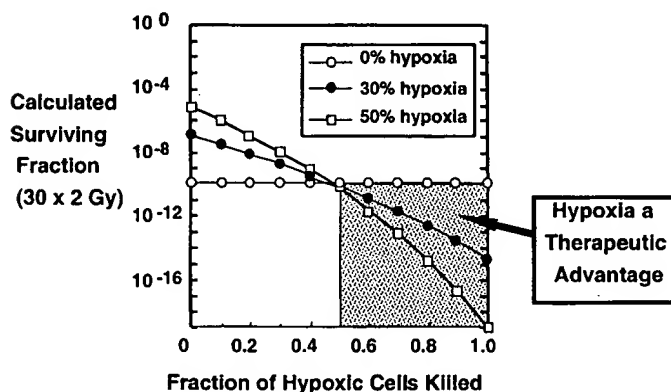


Fig. 6. Results of modeling a fractionated radiotherapy regime of 30 doses of 2 Gy to a tumor with either 0%, 30%, or 50% hypoxic cells with a hypoxic cytotoxin given with each radiation dose. The x axis depicts the fraction of hypoxic cells killed each time the hypoxic cytotoxin is given. In this model, it is assumed that the tumor redistributes its percentage of hypoxic and aerobic cells to the initial value before each radiation and drug delivery. The data show that hypoxia becomes a therapeutic advantage when more than approximately 50% of the hypoxic cells are killed each time the hypoxic cytotoxin is given. This figure was adapted from Ref. 105.

tions to produce equal cytotoxicity, was in the 50–300 range for various cell lines (79). This is to be compared with a HCR of 1–5 for various cell lines for mitomycin C (83, 86–88) and a HCR of 10–20 for nitroimidazoles (89, 90). Fig. 7 shows data for killing of aerobic and hypoxic mouse SCCVII tumor cells by a 1-h exposure to TPZ.

The mechanism of this selective hypoxic cytotoxicity is shown in Fig. 8. TPZ is an excellent substrate for a variety of intracellular reductases that add an electron to the drug, which then becomes protonated to form a neutral radical. This radical is highly reactive and will abstract hydrogen from macromolecules including DNA (if it is close enough), producing both single- and double-strand breaks. We have shown that the hypoxic cytotoxicity of TPZ can be fully accounted for by the double-strand breaks and resulting chromosome breaks that are produced by the drug (91). Under aerobic conditions, oxygen can remove the additional electron from the TPZ radical, thereby back-oxidizing it to the nontoxic parent with a concomitant production of superoxide radical (92). Thus, the differential hypoxic cytotoxicity results from the fact that the TPZ radical is much more cytotoxic than the superoxide radical.

However, there is a problem with this proposed mechanism. One TPZ radical could only be expected to produce one single-strand break, and yet we find some  $10^4$  times more double-strand breaks than could be accounted for by the chance overlap of the single-strand breaks. Our hypothesis to account for this is that the reductase leading to DNA damage is located very close to DNA, thus creating high concentrations

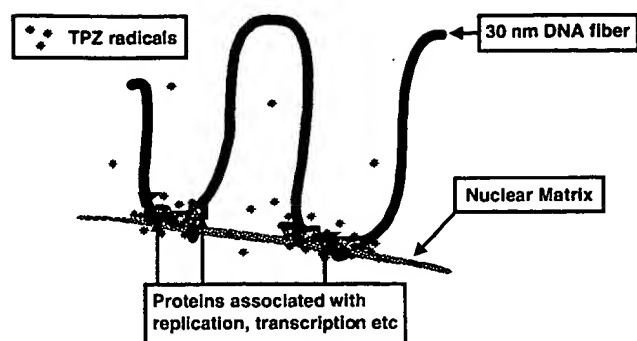


Fig. 9. A model showing the proposed high local concentration of TPZ radicals at the sites of attachment of DNA to the nuclear matrix.

of TPZ radical close to the DNA. We have in fact shown that the activating enzyme(s) leading to DNA damage is (are) located in the cell nucleus (93) and have found that there is a high concentration of TPZ-metabolizing enzymes associated with the nuclear matrix (94). This hypothesis is illustrated in Fig. 9. We propose that the double-strand breaks leading to cell kill under hypoxic conditions are the result of high local concentrations of radicals close to the DNA that is associated with the nuclear matrix. If this is so, a number of phenomena might be predicted. First, because the replication and transcription machinery is associated with the nuclear matrix (95), we might expect major effects of TPZ on replication and transcription. In support of this, we have recently observed very dramatic decreases (of up to 80%) in DNA replication at only moderately toxic TPZ doses under hypoxic conditions.<sup>5</sup> We have also recently shown that the activity of topoisomerase II is dramatically reduced in cells treated with TPZ under hypoxic conditions.<sup>5</sup> This raises a number of interesting possibilities, including the fact that the double-strand breaks induced by TPZ may be the result of poisoning of topoisomerase II, producing cleavable complexes similar to those produced by other topoisomerase II poisons, such as etoposide. This is presently an active area of investigation.

Turning back to the question of activity of TPZ when combined with standard anticancer therapies such as radiation and chemotherapeutic drugs, we have been able to show that TPZ potentiates cell kill by fractionated irradiation at clinically realistic drug and radiation doses (96). Based on these studies, a Phase II multicenter trial of TPZ with irradiation of head and neck tumors was recently reported by the Radiotherapy Oncology Group (97). The results, a 60% local control rate at 18 months for advanced head and neck cancer, are encouraging when compared to the most recent randomized study by the same cooperative group (75).

But the most clinical success has been realized by the combination of TPZ and cisplatin. In testing the concept that TPZ should enhance the antitumor efficacy of a number of anticancer drugs by the complementary cytotoxicity mechanism shown in Fig. 4, we found a remarkable potentiation of cisplatin. Fig. 10 shows the effect of combining a single dose of TPZ with cisplatin on the number of clonogenic cells in 6–8-mm-diameter RIF-1 mouse tumors. In this tumor, TPZ alone produced approximately 0.5 logs of cell kill, cisplatin alone produced approximately 2 logs of cell kills, and the combination of the two drugs given at the same time produced approximately 2.5–3 logs of cell kill. However, when TPZ was given 2–3 h before cisplatin, a large potentiation of cell kill was observed with some 6–7 logs of cell kill produced. Despite this remarkable potentiation of cisplatin toxicity to the tumor cells, we saw no evidence of any increase of cisplatin toxicity to any normal tissues or in its systemic toxicity (98). Although our original hypothesis was that the

interaction of the two drugs would be the result of complementary cytotoxicity [because hypoxic cells in tumors have been shown to be resistant to cisplatin (18, 99)], we have found that this is not the most important mechanism. In fact, there is a specific cellular interaction of TPZ (when given under hypoxia) with cisplatin that mimics the time course and extent of the interaction seen with tumors *in vivo*. We have further shown that this interaction can be accounted for by a delay in the repair of cisplatin-induced interstrand cross-links in cells exposed to TPZ under hypoxia before cisplatin exposure (100). Exactly how TPZ might inhibit or delay the repair of cisplatin-induced interstrand cross-links is presently under investigation.

The promising preclinical data on combining TPZ with cisplatin has led to clinical trials of this combination (101–104). Table 1 shows the results of a Phase III randomized clinical trial in advanced non-small cell lung cancer comparing cisplatin only with the combination of TPZ plus cisplatin (103). In this trial, the overall response rate was doubled by the addition of TPZ to cisplatin, and the median life span was extended significantly. The clinical data also confirmed the preclinical studies showing no potentiation of cisplatin side effects or other toxicities in the combination group. Of note is the fact that there was no increase in hematological toxicity in the combination group, although specific TPZ-associated toxicities of increased nausea and vomiting and muscle cramps were present. These data therefore confirmed the preclinical data that TPZ can produce a tumor-selective potentiation of cisplatin cell kill in human tumors. Because the addition of TPZ to cisplatin is considerably less toxic than the addition to cisplatin of other anticancer drugs such as gemcitabine, navelbine, or Taxol, it is expected that this new bioreductive drug can be added to the best of the current two-drug combinations. To this end, a randomized Phase III trial of cisplatin + navelbine with or without TPZ will soon be started.

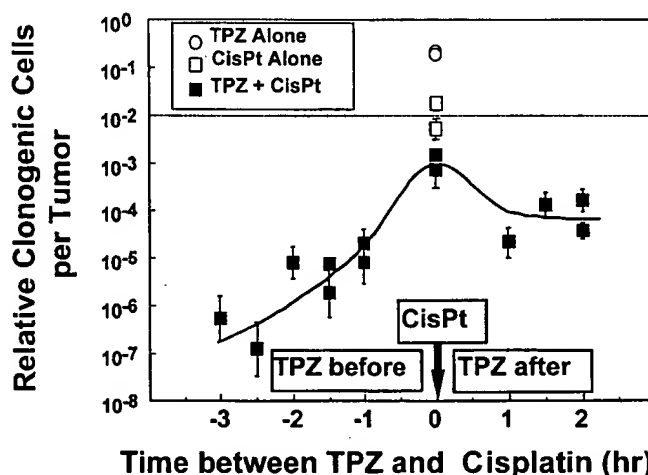


Fig. 10. Cell kill by clonogenic survival in RIF-1 transplanted mouse tumors in mice treated with cisplatin alone (8 mg/kg), TPZ alone (48.6 mg/kg), or the combination of the two. Survival when the two are given simultaneously is consistent with additivity, whereas when TPZ is given before cisplatin, a much greater cell kill is produced in the tumor. This figure was redrawn from Ref. 98.

Table 1 Overall response (partial response + complete response) and median survival time of patients with advanced non-small cell lung cancer treated in a randomized multicenter trial with cisplatin alone or with a combination of TPZ and cisplatin. Data are from Ref. 103.

Treatment	Overall response	Median survival (95% confidence interval)
TPZ + cisplatin (n = 218)	27.5% (21.7–34.0)	34.6 weeks (29.4–39.6)
Cisplatin alone (n = 219)	13.7% (9.4–19.0)	27.7 weeks (24.3–31.3)
P	<0.001	0.0078

<sup>5</sup> K. Peters *et al.*, unpublished observations.

## Summary

The discovery more than 50 years ago that hypoxic cells were resistant to killing by X-rays led to the hypothesis that tumors might be resistant to radiotherapy because their poor blood supply would lead to hypoxia. These early ideas have not only been confirmed in the past 5 years, but tumor hypoxia is now seen as a mechanism for resistance to many anticancer drugs as well as a predisposing factor toward increased malignancy and metastasis. However, tumor hypoxia represents a unique target for cancer therapy that could be exploited for therapeutic benefits. This review focuses on one way in which hypoxia might be exploited: the use of hypoxic cytotoxins, in general, and TPZ, the first purely hypoxic cytotoxin to enter the clinic, in particular. However, this is only one possibility. Other approaches being investigated by different groups are targeting some of the specific hypoxia-induced proteins such as HIF-1 or using hypoxia to obtain tumor-specific gene expression for gene therapy.

## Acknowledgments

Many people have contributed to both the ideas and data that I have presented in this review, and my sincere thanks go to them. In particular, I owe a debt of gratitude to Dr. William Lee, whose chemistry group teamed with my laboratory for many years to develop new anticancer drugs based on tumor hypoxia. In recent years, his position was filled ably by Dr. Michael Tracy, who has contributed to the clinical development of TPZ by synthesizing many analogues. I am also greatly indebted to members of my own laboratory, past and present. Past members include Dr. Elaine Zeman, who performed many of the original cellular studies with TPZ, Dr. Jingli Wang, whose work on chromosome aberrations established the importance of this lesion for TPZ cytotoxicity, and for Drs. Margaret Baker, Edward Bump, Amato Giaccia, James Elwell, Andrew Minchinton, Bronwyn Siim, and Brad Wouters, who contributed a lot to the understanding of the mechanism and clinical potential of TPZ. I am also grateful to present laboratory members Yvette Delahoussaye, Mary Jo Dorie, James Evans, Zelanna Goldberg, Mary Kovacs, Doug Menke, Patricia McAfee, and Katherine Peters for their valuable contributions.

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